Identification and transcriptional profiling of *Pseudomonas putida* genes involved in furoic acid metabolism

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Abstract

Pseudomonas putida Fu1 metabolizes furfural through a pathway involving conversion to 2-oxoglutarate, via 2-furoic acid (FA) and coenzyme A intermediates. Two *P. putida* transposon mutants were isolated that had impaired growth on furfural and FA, and DNA flanking the transposon insertion site was cloned from both mutants. The transposons disrupted *psfB*, a LysR-family regulatory gene in mutant PSF2 and *psfF*, a GcvR-type regulatory gene in PSF9. Disruption of two genes adjacent to *psfB* demonstrated that both are important for growth on FA, and ORFs in the proximity of *psfB* and *psfF* were transcriptionally activated during growth of *P. putida* on FA. Transcript levels increased in response to FA by 10-fold (a putative permease gene) to > 1000-fold (a putative decarboxylase gene). The LysR-family gene appears to act positively, and the GcvR-family gene negatively, in regulating expression of neighboring genes in response to FA.

Introduction

Furan-degrading microorganisms occur naturally where decaying plant materials are present. Bacteria and fungi degrade furans including furfural, 5-hydroxymethylfurfural, furfuryl alcohol, and furoic acid (FA) (Brune *et al.*, 1983; Trudgill, 1984; Folkerts *et al.*, 1989; Boopathy *et al.*, 1993; Wang *et al.*, 1994; Boopathy, 1996, 2002; Belay *et al.*, 1997; López *et al.*, 2004). Some microorganisms cannot grow on furans but transform them (Trudgill, 1984; Gutiérrez *et al.*, 2006; Petersson *et al.*, 2006), presumably to detoxify the compounds.

Furan degradation in *Pseudomonas putida* has been characterized, and some enzymes have been purified (Kitcher *et al.*, 1972; Trudgill, 1984; Koenig & Andreesen, 1989, 1990). Aerobic growth involves oxidation of furfuryl alcohol and furfural to FA. After thioesterification of FA, a molybdenum-containing dehydrogenase converts 2-furoyl-CoA to 5-hydroxy-2-furoyl-CoA. Modification of the furan ring results in conversion to α-ketoglutaric acid. To date, there is no genetic understanding of the pathway. To begin to understand the genetic basis for furan metabolism, we isolated mutants of *P. putida* that are impaired in growth on FA. Two disrupted genes, both apparent transcriptional regulators, were used as a starting point to identify neighboring genes involved in FA metabolism.

Materials and methods

Plasmids, bacterial strains and growth conditions

Plasmids and strains are described in supplementary Table S1. *Escherichia coli* was grown at $37\,^{\circ}C$ on Luria–Bertani (LB) medium. *Pseudomonas putida* was cultured at $30\,^{\circ}C$ in defined mineral medium [MM; pH 6.8; 25 mM KH_2PO_4, 25 mM Na_2HPO_4, 0.1% $(NH_4)_2SO_4$, 0.1% Hutner mineral base (Gerhardt *et al.*, 1981)]. Liquid cultures were shaken at 250 r.p.m. (Innova 4230 incubator shaker, New Brunswick, Edison, NJ). Solid LB medium contained 1.5% (w/v) Bacto agar. Solid MM contained 1.5% (w/v) Noble agar (Becton, Dickinson, Sparks, MD), sterilized separately in water.

Carbon sources were sterilized separately and added to media at 5 mM, except succinate, which was 10 mM. Growth was initiated with a 2% (v/v) inoculum grown overnight in succinate MM. Generation times were determined by

measuring OD of MM cultures (600 nm, 1 cm pathlength, DU 640 spectrophotometer, Beckman Instruments, Fullerton, CA) in 10-mL Erlenmeyer flasks.

Molecular biological methods

Molecular biological techniques were performed using standard methods (Sambrook & Russell, 2001). Where stated, genomic DNA was amplified by arbitrary PCR (Das *et al.*, 2005) using a primer of known sequence paired with an arbitrary primer (5′-GGCCACGCGTCGACTAGTACN$_{10}$AC GCC-3′). A primer identical in sequence to the 5′ end of the arbitrary primer was used to reamplify products in a second PCR.

RNA was isolated from cultures in early log phase (3–4 h growth) in MM containing the specified carbon source(s). 7.5×10^8 cells were treated with RNAprotect Bacteria Reagent, followed by RNA isolation using the RNeasy Plus kit (Qiagen, Valencia, CA). RNA was quantified using a ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Aliquots were diluted to *c.* 150 ng mL^{-1} and treated with Turbo DNAse (Ambion, Austin, TX).

Quantitative PCR

DNAse-treated RNA was repurified and assessed (2100 bioanalyzer Lab-on-Chip, Agilent, Wimington, DE) before real-time quantitative reverse transcriptase (qRT)-PCR analysis. Primers and probes are described in supplementary Table S2 and Fig. 1. Assays used RT-PCR master mix (Qiagen) and a Rotorgene 6000 (Corbett Life Science, Sydney, Australia). RT (50 °C for 30 min) and denaturation (95 °C for 15 min) were followed by 40 amplification–detection cycles (95 °C for 15 s and 60 °C for 60 s). Standard curves using linearized plasmid DNA yielded r^2 values

> 0.99 and efficiencies of 0.89–1.07. No signal was detected in control reactions lacking template or RT. All assays were performed in triplicate.

Bacterial conjugation and mutagenesis

Donor and recipient strains were mixed (1 : 5 ratio) on LB agar, incubated overnight at 30 °C, and transferred to MM agar containing succinate and selective antibiotic. For transposon mutagenesis, *P. putida* Fu1 was mated with *E. coli* S17-1λpir(pUTminiTn5-Km) to generate a library of mutant strains. The mixture was diluted onto MM agar containing FA plus a low (1 mM) concentration of succinate. After 24 h, small colonies were identified as potential FA nonmetabolizing mutants.

Specific genes were disrupted with a kanamycin (km) cassette and transferred by conjugation into wild-type (WT) *P. putida*. After antibiotic selection to ensure the presence of the plasmid, each recombinant strain was subcultured in antibiotic-free succinate MM. Then, colonies were replica plated to identify strains which had retained kanamycin-resistance but lost resistance to the vector-encoded antibiotic. These strains were presumed to have undergone a double cross-over event. The presence of the disrupted gene on the chromosome, in place of the WT copy, was verified by PCR amplification across the insertion site.

Results and discussion

Isolation of FA mutants

In a screen of transposon mutants, PSF2 and PSF9 formed small colonies compared with WT strain Fu1 on solid MM containing limiting (1 mM) succinate and FA at a concentration (5 mM) adequate for WT growth. Further

Fig. 1. Map of DNA cloned from furoic acid growth mutants PSF2 and PSF9. Sites of minitransposon insertion are indicated by arrowheads. ORFs and direction of transcription are indicated by arrows, and similarity to genes of known function is given. Probes used for qRT-PCR are shown as black bars. Dashed arrows indicate truncated genes.
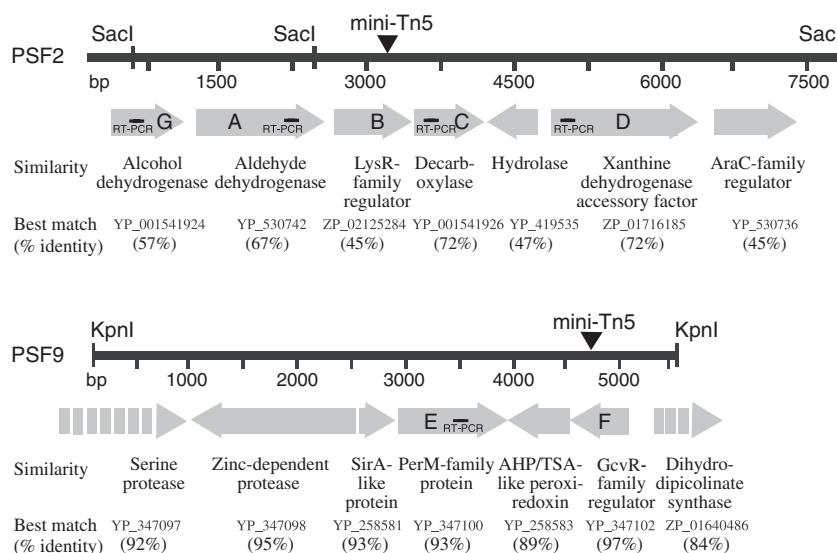
Table 1. Growth of *Pseudomonas putida* Fu1 and mutants*

		Carbon source		
Strain	Description	Furoic acid	Succinate	4-Hydroxy-benzoate
Fu1	WT	121 ± 10	82 ± 5	100 ± 12
PSF2	*psfB-*	–	85 ± 4	101 ± 17
PSF2(pPSF210)	*psfB* in trans	424 ± 27	ND	ND
PSF7	*psfB-*	–	80 ± 8	102 ± 11
PSF9	*psfF-*	209 ± 65	85 ± 5	103 ± 10
PSF11	*psfA-*	∞	80 ± 6	100 ± 14
PSF11(pPSF237)	*psfA* in trans	460 ± 90	ND	ND
PSF12	*psfD-*	598 ± 162	74 ± 6	95 ± 9
PSF12(pPSF228)	*psfD* in trans	276 ± 68	ND	ND

*Generation time (min); values are averages of at least three growth experiments each performed in duplicate.
–, no growth initially, but growth with doubling time of 11–15 h began after incubation for several days; ND, not done; ∞, no growth.

examination showed that mutant PSF2 did not grow on FA, and mutant PSF9 grew more slowly than WT on FA (Table 1). Because both mutants grew at WT rates on succinate and on 4-hydroxybenzoate, the mutations are not general growth defects.

Identification of genes disrupted in FA-utilization mutants

DNA containing the minitransposon insertion in PSF2 and PSF9 was cloned and sequenced (GenBank # EU290170). Restriction fragments suitable for cloning were identified by Southern analysis of genomic DNA, using a kanamycin gene probe. DNA flanking the insertion site was obtained from each mutant (Fig. 1) by selecting for kanamycin-resistant recombinant plasmids. The 5 kb of sequence obtained from PSF2 was extended *c.* 3 kb upstream of the original SacI-cloning site by cloning partially digested genomic DNA, and by arbitrary PCR. The sequences at the insertion sites suggested that both were in regulatory genes (Fig. 1). The gene in PSF2, designated *psfB*, is similar to LysR-family regulatory genes. LysR regulators typically activate transcription in response to an inducing molecule (Schell, 1993). In PSF9, the insertion site, *psfF*, is homologous to GcvR-family genes, which often regulate metabolism via gene repression (Heil *et al.*, 2002).

Comparison to known sequences allowed tentative description of several additional ORFs (Fig. 1). Sequence from PSF9 is similar to genomes from *Pseudomonas* species. In fact, the entire 5689-bp nucleotide sequence of the PSF9 cluster is 82% identical to a region of the *Pseudomonas fluorescens* PfO-1 genome (GenBank CP000094). Deduced amino acid sequences in the cloned region are *c.* 90% identical to those from other pseudomonads (Fig. 1),

indicating a possible shared function related to stress tolerance. PsfE is similar to the PerM subfamily, which may function as solute transporters. Other predicted gene products include two proteases possibly having a stress response or chaperone function, a SirA-family two-component response regulator, and a peroxidase from the AhpC/thiol-specific antioxidant (TSA) family. TSA proteins have been shown to protect against oxidative damage of DNA. All of these functions are presumably relevant for growth on furans (Zaldivar *et al.*, 1999).

The best matches to sequences from mutant PSF2 were identified in diverse bacteria including *Rhodopseudomonas* and *Magnetospirillum* (Fig. 1). Some ORFs are similar to genes encoding activities proposed for furan metabolism. PsfG and PsfA are of interest in the context of furfuryl alcohol and furfural degradation, which occurs through oxidation to FA and subsequent conversion of furoyl-coenzyme A to 5-hydroxy-furoyl-coenzyme A (Trudgill, 1984; Koenig & Andreesen, 1990). PsfG is a hypothetical 26.0-kDa short chain dehydrogenase in the FabG 3-ketoacyl-ACP reductase family. PsfA (51.0 kDa) is similar to NAD-dependent succinate semi-aldehyde dehydrogenases.

The deduced amino acid sequence of PsfC (22.7 kDa) is similar to 3-octaprenyl-4-hydroxybenzoate carboxy-lyases (UbiX) for ubiquinone biosynthesis (Gulmezian *et al.*, 2007), and to related aromatic acid decarboxylases. However, PsfC is not similar in size or sequence to nonoxidative aromatic acid decarboxylases (Lupa *et al.*, 2005), a group of enzymes that includes pyrrole-2-carboxylate decarboxylase from *Bacillus megaterium* (Omura *et al.*, 1998). A hydrolase function has been proposed in the pathway for FA degradation, and a putative hydrolase gene was identified adjacent to *psfC*. In addition, an ORF similar to the xanthine dehydrogenase maturation factor of *Pseudomonas aeruginosa* was identified. The ORF, designated *psfD*, is noteworthy because FA degradation in *P. putida* Fu1 proceeds via 2-furoyl-coenzyme A dehydrogenase, a molybdoenzyme similar to xanthine dehydrogenase (Koenig & Andreesen, 1989, 1990).

Mutant growth phenotypes

WT *P. putida* has a 121 min generation time in liquid MM containing FA as sole carbon source. In comparison, PSF9 has a doubling time of *c.* 209 min. PSF2 did not initially grow on FA, but began to grow after incubation for several days. To confirm that the FA-minus phenotype was due to mutation in *psfB*, the mutation was reconstructed in Fu1. The resulting strain, PSF7, and the original *psfB* mutant PSF2 both had doubling times of *c.* 11–15 h on FA, suggesting a second-site mutation occurs and compensates for the function(s) lost with disruption of *psfB*. Complementation *in trans* with *psfB* restored the ability of PSF2 to

grow on FA (Table 1). The results of these growth and complementation studies confirmed the involvement of *psfB* in FA metabolism.

Two ORFs identified near *psfB* (Fig. 1) were examined for a potential role in growth on FA. Mutations were constructed in *psfA* and *psfD* by inserting a kanamycin-resistance gene cassette and replacing the WT copy of the gene by cross-over on the Fu1 chromosome. Both the *psfA* and *psfD* mutants (PSF11 and PSF12, respectively) were deficient in growth on FA (Table 1). PSF11 did not grow on FA, whereas PSF12 grew more slowly than WT. Reconstitution of intact *psfA in trans* restored the ability of PSF11 to grow on FA, although more slowly than WT. The ability of PSF12, a *psfD* mutant, to grow to some extent on FA implies existence of an additional gene encoding a similar activity. For example, the *P. putida* xanthine dehydrogenase also catalyzes the furoyl-coenzyme A dehydrogenase reaction (Koenig & Andreesen, 1990). Improved growth of PSF12 was restored by *psfD in trans*.

Quantitation of transcripts

Growth-deficient phenotypes associated with disruptions in two putative regulatory genes, *psfB* in PSF2 and *psfF* in PSF9, led us to examine transcriptional regulation of neighboring genes. Previously, furfuryl alcohol- and furfural dehydrogenase activities were shown to be induced in *P. putida* by furfural but not FA (Koenig & Andreesen, 1990). Genes and enzymes involved in degradation of tetrahydrofuran are specifically induced in *Pseudonocardia* (Thiemer *et al.*, 2003), and aldehyde dehydrogenases are induced during growth of *Ralstonia eutropha* on tetrahydrofurfuryl alcohol (Schräder *et al.*, 2001). Expression of five genes was measured in WT *P. putida* Fu1 by qRT-PCR (Fig. 2). Consistent with a role in FA metabolism, transcript levels increased for all five genes when Fu1 was grown on FA. The greatest relative increase was observed for *psfC*; cells grown on FA produced c. 1000-fold more *psfC* transcript than cells grown on succinate. Growth on FA resulted in a 10-fold increase of *psfE*, which presumably encodes a membrane protein.

To determine whether gene expression is dependent on *psfB* and *psfF*, qRT-PCR was also performed on RNA from PSF2 and PSF9 (Fig. 3). Cells were grown on succinate (noninducing medium) or a mixture of succinate and FA (inducing medium; succinate was included to support growth of mutants and was also added to WT cultures). In *psfB* mutant PSF2, transcription of *psfC* and *psfD* was not induced (Fig. 3a). Activation of the two genes requires *psfB*, a result consistent with the activation function frequently assigned to LysR-family regulators (Schell, 1993). However, induction patterns for *psfA* and *psfG* in PSF2 were similar to WT, indicating that transcription of *psfA* and *psfG* does not require *psfB*.
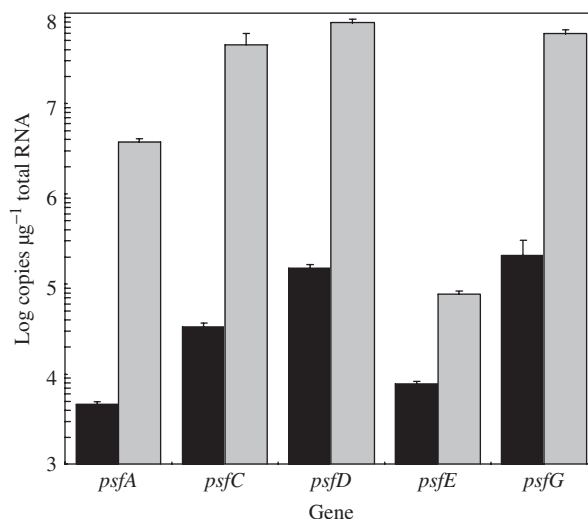
Fig. 2. Expression of several cloned genes increases in response to growth on furoic acid. Gene copy numbers were determined by qRT-PCR as described in Materials and methods. Black bars show transcripts from Fu1 cells grown on succinate, gray bars are results for cells grown on furoic acid. Error bars indicate SE from a minimum of three replicates.

Figure 3b shows that *psfF* negatively regulates expression of *psfE*. The *psfE* copy number in PSF9 cells grown on succinate was similar to the induced level observed in cells grown on succinate plus FA. This data is consistent with the function of GcvR-family proteins as transcriptional repressors (Heil *et al.*, 2002). Because *psfE* is regulated in response to FA (Fig. 2), the *psfE*-encoded protein likely plays a role in growth on FA. However, the growth phenotype of mutant PSF9 cannot be attributed to lack of *psfE* gene expression (Fig. 3b). Slower growth of PSF9 may instead be caused by an indirect effect of *psfF* on another gene(s).

Genes activated by growth on FA would be expected to also be induced by furfuryl alcohol or furfural, because dissimilation of the alcohol and aldehyde progresses through FA (Kitcher *et al.*, 1972; Koenig & Andreesen, 1989, 1990). Thus, FA (or other inducing metabolite) would be present in cells grown on furfuryl alcohol or furfural. As expected, *psfA* transcription was induced in response to furfural and furfuryl alcohol (Fig. 3a). The *psfA* transcript increased 15- and 7-fold, respectively, in WT cells grown in medium containing succinate plus furfural or furfuryl alcohol, compared with 277-fold in cells grown on succinate plus FA. Induction of *psfA*, in response to the alcohol and aldehyde, may be due to a need for furfural to be quickly metabolized in order to mitigate toxicity of the aldehyde. In contrast to results obtained for *psfA*, the level of *psfC*, *psfD*, *psfE*, and *psfG* transcripts did not increase appreciably in cells grown on succinate plus furfuryl alcohol or furfural. The differences observed in gene expression under these conditions suggest that additional factors or metabolites may be involved in their regulation.
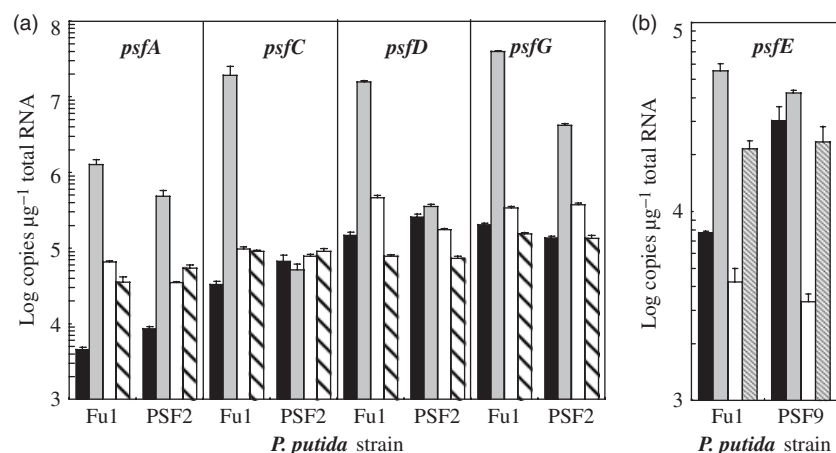
Fig. 3. qRT-PCR analysis of gene expression in WT *Pseudomonas putida* Fu1, *psfB* mutant PSF2, and *psfF* mutant PSF9. In these experiments, early-log-phase cultures in succinate MM were divided and an additional carbon source was added. One culture, an uninduced control, received succinate (black bars), while others received one of the following: furoic acid (gray bars), furfural (white bars), or furfuryl alcohol (hatched bars). RNA was isolated 3–4 h after addition of the new carbon source. SE is indicated for a minimum of three replicates. (a) Comparison of *psfA*, *psfC*, *psfD*, and *psfG* transcript copy numbers in WT *P. putida* Fu1 and PSF2. (b) Comparison of *psfE* transcript copy number in Fu1 and PSF9.

Taken together, qRT-PCR and phenotype analyses indicate that growth on FA is inducible in *P. putida*, and point to a role in furan dissimilation for several cloned genes. Two genes, *psfB* and *psfF*, are similar to known regulatory genes. Mutations caused *P. putida* to grow slowly (*psfF*-) or not at all (*psfB*-) on FA (Table 1), and exhibit altered transcript levels of neighboring genes (Fig. 3). Mutants in two additional genes, *psfA* and *psfD*, display impaired growth on FA. Transcription of five genes increases in response to growth on FA (Fig. 2), and transcription of *psfA* is also induced in response to furfuryl alcohol and furfural (Fig. 3). The *psf* genes are subject to both positive (*psfB*) and negative (*psfF*) control. PsfB activates transcription of two genes (*psfC* and *psfD)*, while PsfF represses expression of *psfE*. Identification of genes regulated in response to, and required for growth on, FA provides an initial basis for understanding FA metabolism in bacteria.

Acknowledgements

Statement

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Supplementary material

The following material is available for this article:

Table S1. Bacterial strains and plasmids

Table S2. Primers and probes used for qRT-PCR

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1574-6968.2008.01196.x (This link will take you to the article abstract).

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